PHYLOGENY OF THE *SEPIA PHARAONIS* SPECIES COMPLEX (CEPHALOPODA: SEPIIDA) BASED ON ANALYSES OF MITOCHONDRIAL AND NUCLEAR DNA SEQUENCE DATA

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(Received 13 May 2010; accepted 27 September 2010)

ABSTRACT

The pharaoh cuttlefish, *Sepia pharaonis* Ehrenberg, 1831, is a commercially fished species found from Japan to East Africa. Previous morphological and genetic work (the latter based on the 16S rRNA mitochondrial gene) suggested that *S. pharaonis* is a species complex, but relationships within the complex remained unresolved. To clarify these relationships, we have sequenced an additional mitochondrial gene region (cytochrome oxidase subunit I) and a nuclear gene region (rhodopsin) from over 50 specimens from throughout the range of *S. pharaonis*. We have also added sequence data from two specimens of *Sepia ramani* Neethiselvan, 2001, collected in southeastern India. *Sepia ramani* is a species that is morphologically very similar to *S. pharaonis*, and there is some question regarding its status as a distinct species. Phylogenetic analyses of a dataset comprising all three-gene regions revealed a monophyletic *S. pharaonis* complex consisting of a western Indian Ocean clade, a northeastern Australia clade, a Persian Gulf/Arabian Sea ('Iranian') clade, a western Pacific clade and a central Indian Ocean clade. Relationships among these clades remain somewhat poorly supported except for a clade comprising the Iranian clade, the western Pacific clade and the central Indian Ocean clade. One *S. pharaonis* specimen was collected in the Arabian Sea, but was found to be a member of the western Indian Ocean clade, suggesting that gene flow between these regions has either occurred recently or is ongoing. Both specimens of *S. ramani* are members of the *S. pharaonis* complex, but their mtDNA haplotypes are not closely related – one is a member of the central Indian Ocean clade, while the other is rather distantly related to the northeastern Australia clade. We suggest that '*S. pharaonis*' may consist of several species, but morphological work is needed to clarify species-level taxonomy within this complex.

INTRODUCTION

The pharaoh cuttlefish *Sepia pharaonis* Ehrenberg, 1831 (Sepiidae) is a broadly distributed neritic demersal cephalopod species found from East Africa to southern Japan. *Sepia pharaonis* is a commercially harvested species, and it is a significant component of cephalopod fisheries throughout its range (Nesis, 1987; Reid, Jereb & Roper, 2005). Reid *et al.* (2005) noted that *S. pharaonis* is the most common species of cuttlefish caught in the Persian Gulf, the Gulf of Oman, the Andaman Sea, the Gulf of Thailand, the Philippines and along the southern coast of China, and Nesis (1987) wrote that "[*Sepia pharaonis*] is the most important object of the cuttlefish fishery in the northern part of the Indian Ocean and southeastern Asia". *Sepia pharaonis* has also been proposed as a promising species for mariculture due to its high spawning success, rapid rate of growth, disease resistance and tolerance of crowding and handling (Minton *et al.*, 2002; Barord, Keister & Lee, 2010).

Though molecular genetic data are scarce for many invertebrate fisheries in part due to the small, local scale of many such fisheries (Thorpe, Sole-Cava & Watts, 2000), several cephalopods are targets of large-scale fisheries, and population genetic studies have been published for a number of these (e.g. Adcock *et al.*, 1999; Shaw, Pierce & Boyle, 1999; Kassahn *et al.*, 2003; Shaw *et al.*, 2004; Perez-Losada *et al.*, 2007). Despite the commercial importance of *S. pharaonis*, very little has been published on the phylogeography or population genetics of this species, presumably due in part to its broad geographic distribution. Such data could be particularly important for *S. pharaonis*. The broad coastal distribution of this species across biogeographic zones, coupled with the high incidence of cryptic speciation in commercially fished marine invertebrates

(including some cephalopods; Yeatman & Benzie, 1994), suggests the presence of multiple stocks. Furthermore, the semelparous annual life cycle of many cephalopods (probably including *S. pharaonis*; Gabr *et al*., 1998, but see Aoyama & Nguyen, 1989) makes their stocks highly vulnerable to overexploitation (Thorpe *et al*., 2000).

There is some morphological and behavioural evidence that *S. pharaonis* may be a complex of closely related species. Norman (2000) suggested that *S. pharaonis* comprises three forms: *S. pharaonis* (*s. s.*) (found in the western Indian Ocean from the Red Sea to the Persian Gulf; the eastern limit is unknown); *S.* '*pharaonis* II' (Japan to the Gulf of Thailand, Philippines and north Australia) and *S.* '*pharaonis* III' (Maldives to Andaman Sea coast of Thailand). There appear to be consistent reproductive differences among these three forms. While mating, *S. pharaonis s. s.* males show zebra lines on the third arm pair, while *S. pharaonis* II males have broken lines and *S. pharaonis* III males have spots (Norman, 2000). In addition, *S. pharaonis s. s.* spawns between August and October, while *S. pharaonis* II (in Hong Kong) spawns from March through May and *S. pharaonis* in India spawns all year round (Norman, 2000). There are hints that this complex may consist of more than three species; for example, hectocotylus morphology differs between males collected in Japan and Australia (Reid *et al*., 2005).

The status of *S. pharaonis* is further complicated by the recent description of a new species, *S. ramani* Neethiselvan, 2001 that appears to be closely related to *S. pharaonis*. *Sepia ramani* is so far only known from the Gulf of Mannar in southeastern India (Neethiselvan, 2001). Reid *et al*. (2005) depict the distribution of this species as including the coast of Sri Lanka and the southwest coast of India, but this appears to be an error (A. Reid, personal communication). Neethiselvan (2001) noted that *S. ramani* is difficult to distinguish from *S. pharaonis*, although he listed some characters that allow the two species to be identified: *S. ramani* has 5–6 enlarged club suckers, with 3–4 greatly enlarged, whereas *S. pharaonis* has 15–24 enlarged suckers, all of approximately equal size; and there are 14–16 transverse rows of normal suckers on the hectocotylus of *S. ramani*, but only 10–12 such rows in *S. pharaonis*. Neethiselvan (2001) also noted that some morphometric characters (cuttlebone width, inner cone length and tentacular club length) could be useful for distinguishing between the species, but there is some overlap between the two species in all of these characters. The strong morphological similarities suggest a close relationship between *S. pharaonis* and *S. ramani*, but the nature of this relationship is unknown. *Sepia ramani* could be a close relative of the *S. pharaonis* complex, it could be a genetically distinct subclade (or species) within that complex or it could represent aberrant specimens of *S. pharaonis*.

Attempts to untangle this putative species complex using molecular genetic data have been limited to a study by Anderson *et al*. (2007) based on partial mitochondrial 16S rRNA sequence data collected from *S. pharaonis* samples from throughout the Indian Ocean and western Pacific Ocean. Anderson *et al*. (2007) found that *S. pharaonis* comprises five distinct clades: a western Indian Ocean clade (Gulf of Aden and Red Sea), a northeastern Australia clade, an Iranian clade (northern Gulf of Oman and the Persian Gulf), a central Indian Ocean clade (India and the Andaman Sea coast of Thailand) and a western Pacific clade. In this study, we build upon Anderson *et al*.'s (2007) dataset by (1) adding another mitochondrial gene region and a nuclear gene region in an effort to clarify relationships among subclades of *S. pharaonis* and test monophyly of the *S. pharaonis* complex, and (2) by expanding the taxon sample to include specimens of *S. ramani*.

MATERIAL AND METHODS

Tissue sample collection, DNA extraction and sequencing

Tissue samples were collected from *Sepia pharaonis* individuals from throughout the range of the species (Table 1, Fig. 1) and shipped to the first author (F.E.A.) in 80–100% EtOH as part

Table 1. Collection locality and GenBank accession data for all specimens of *Sepia pharaonis* complex.

Locality	GenBank accession numbers		Latitude	Longitude
	COI	Rhodopsin		
Red Sea (Yemen)	HM164520–HM164523, HM164536	HM164477–HM164480, HM164483	15°46′N	42°37′E
Gulf of Aden (Yemen)	HM164519, HM164524, HM164525, HM164527, HM164536	HM164476, HM164481, HM164482, HM164484	12°44′N	44°40′E
Persian Gulf (Iran)	HM164528	HM164485	28°40′N	50°45′E
South Gulf of Oman (Oman)*	HM164534	–	–	–
North Gulf of Oman (Iran)	HM164505, HM164506, HM164537, HM164538	HM164462, HM164463	25°08′N	60°21′E
Kochi (India)	HM164489, HM164491, HM164492, HM164532, HM164533	HM164446, HM164448, HM164449	9°55′N	76°05′E
Tuticorin (India)	HM164515, HM164518	HM164472, HM164475	~8°48′N	~78°09′E
Veraval (India)	HM164490, HM164516, HM164531	HM164447, HM164473, HM164488	~20°N	~70°E
Vishakapatanam (India)	HM164500, HM164501, HM164517	HM164457, HM164458, HM164474	17°41′N	83°18′E
Phuket (Thailand)	HM164508–HM164513, HM164535	HM164465–HM164470	~7°N	~98°E
Chumphon (Thailand)	HM164493, HM164494, HM164502, HM164503	HM164450, HM164451, HM164459, HM164460	~10°N	~99°E
Prachuap Khiri Khan (Thailand)	HM164499, HM164514	HM164456, HM164471	11°48′N	100°5′E
Gulf of Carpentaria (Australia)	HM164495–HM164497, HM164504	HM164452– HM164454, HM164461	12°S	141°E
Northeast Queensland (Australia)	HM164498, HM164507	HM164455, HM164464	18°25′S	146°28′E
Sepia ramani (Tuticorin, India)	HM164529, HM164530	HM164486, HM16487	~8°48′N	~78°09′E

Abbreviation: ~, approximate values denoting collections from several sites in close proximity to one another. *Latitude and longitude not available, collected near Muscat, Oman.

Figure 1. Map showing the type localities for *Sepia pharaonis* (*) and sampling localities, modified from Anderson *et al.* (2007): 1, Red Sea; 2, Gulf of Aden; 3, Persian Gulf (Iran); 4, northern Gulf of Oman (Iran); 5, southern Gulf of Oman (Oman); 6, Veraval; 7, Kochi; 8, Tuticorin; 9, Vishakapatanam; 10, Phuket; 11, Prachuap; 12, Chumphon; 13, Taiwan; 14, Gulf of Carpentaria; 15, northeast Queensland.

of an earlier study (Anderson *et al.*, 2007). Tissue samples from two additional specimens of *S. pharaonis* and two specimens of *S. ramani* were collected in Tuticorin, India, in October 2007. DNA extraction, PCR product purification, automated DNA sequencing and sequence editing were as described in Anderson *et al.* (2007). PCRs were performed using a Perkin-Elmer 9700 thermal cycler. A fragment of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene and a fragment of the rhodopsin gene were amplified using universal metazoan COI PCR primers (Folmer *et al.*, 1994) and cephalopod-specific rhodopsin PCR primers (Strugnell *et al.*, 2005), and HotStar Master Mix (QIAGEN) following manufacturer's protocols (half-reactions). Thermal cycling regimes were as follows: 94° (1 min) – 42° (1 min) – 68° (1:30), repeated for 35 cycles, with a 7-min terminal extension step at 72° (COI); 94° (1 min) – 42° (1 min) – 72° (1:30), repeated for 35 cycles, with a 7-min terminal extension step at 72° (rhodopsin).

Sequence alignment and phylogenetic analyses

COI and rhodopsin sequences obtained in this study were combined with all available sepiid 16S rRNA, COI and rhodopsin sequences in GenBank (http://www.ncbi.nlm.nih.gov/) as of 17 February 2009. Alignment of the COI and rhodopsin sequences was performed by eye in Se-Al v. 2.0a11 (Rambaut, 2002). Several phylogenetic analyses were performed for the COI and rhodopsin data individually and for three combinations of data – one consisting of the combined mtDNA data only (i.e. COI sequences from this study plus 16S rRNA sequences from Anderson *et al.*, 2007) and two 'three-gene analyses' (comprising all COI, 16S rRNA and rhodopsin sequences generated here and in Anderson *et al.*, 2007). Only 16S rRNA sequences are available from the two specimens of *S. pharaonis* from Taiwan. Sequence data for all outgroups were downloaded from GenBank. For the combined mtDNA dataset, all sepiids for which both COI and 16S rRNA sequences were available in GenBank were included as outgroups to provide as robust a test as possible for *S. pharaonis* monophyly (Table 2). For the three-gene dataset, GenBank data for COI, 16S rRNA and rhodopsin were only available for three outgroup species (two sepiids – *Sepia officinalis* and *Metasepia tullbergi* – and one sepiolid, *Euprymna scolopes*), so two analyses were performed – one in which only these three taxa were used as outgroups and one in which all sepiid and

Table 2. GenBank accession numbers for all outgroup taxa.

Species name	GenBank accession numbers		
	16S rRNA	COI	Rhodopsin
Sepia aculeata	AF369113 (Z)	AF350494 (Z)	–
S. elegans	AY293657 (N)	AY293707 (N)	–
S. esculenta	AF369115 (Z)	AF359554 (Z)	–
S. hierredda	AY368675 (M)	AJ583493 (M)	–
S. kobiensis	AB192323 (T)	AB193813 (T)	–
S. latimanus	AF369116 (Z)	AY185506 (Z)	–
S. lycidas	AB192321 (T)	AB192337 (T)	–
S. madokai	AB192320	AB192336	–
S. officinalis	AB193804 (Y)	AB193812 (Y)	AF000947 (B)
S. pardex	AB193801 (Y)	AB193809 (Y)	–
S. peterseni	AB192324 (T)	AB192339 (T)	–
S. pharaonis	AF369117 (Z)	AF359555 (Z)	–
S. robsoni	AF369957 (Z)	AF350495 (Z)	–
Euprymna scolopes	AY293663 (N)	AY293712 (N)	AY616923 (S)
Metasepia tullbergi	AB192325 (T)	AB192340 (T)	AY616925 (S)
Semirossia tenera	AY426435 (N)	AY426436 (N)	–
Sepiella maindroni	AB192326 (T)	AB192341 (T)	–

Codes in parentheses refer to original studies (B, Bellingham, Morris & Hunt, 1998; Murphy, J.M., Hernandez, M.N., Pereles-Raya, C. and Balguerias, E. unpubl.; N, Nishiguchi, Lopez & Von Boetzky, 2004; S, Strugnell *et al.*, 2005; T, Takumiya *et al.*, 2005; Y ,Yoshida, Tsuneki & Furuya, 2006; Zheng, X.D., Wang, R.C., Xiao, S. and Chen, B. unpubl.). Rhodopsin sequences for *E. scolopes*, *M. tullbergi* and *S. officinalis* are from different individuals than the mitochondrial sequences. Sequences for *S. madokai* have been removed from GenBank subsequent to the completion of the analyses described in this paper.

sepiolid taxa used in the combined mtDNA data analyses were used as outgroups.

Maximum parsimony (MP) bootstrap and Bayesian analyses were performed for each dataset in PAUP* v. 4.0b11 (Swofford, 2002) and MrBayes v. 3.1.1 (Ronquist & Huelsenbeck, 2003). Due to the size and composition of the dataset, analyses of each MP bootstrap pseudoreplicate resulted in thousands of equally parsimonious trees. To facilitate the analysis, 100 bootstrap pseudoreplicates were analysed, with the maximum number of trees retained set to 10,000 (maxtrees = 10,000) and a heuristic search with the following parameters: 100 random-addition-sequence replicates (addseq = random nreps = 100), holding 10 trees at each step (hold = 10), retaining only 100 trees of length $\geq$1 per replicate (nchuck = 100, chuckscore = 1). Ten such analyses were run, with bootstrap support values for each node averaged across all 10 runs.

Multiple data partitioning schemes were tested for Bayesian analyses of the separate and combined datasets. For the 16S rRNA dataset, the data were not partitioned (i.e. a single substitution model was used for the dataset). For the protein-coding gene datasets, the data were either not partitioned or partitioned by codon position (with a separate substitution model for each codon position and model parameters estimated separately for each partition). Best-fitting DNA substitution models for each partition were chosen by estimating a neighbour-joining tree for the partition using Jukes–Cantor distances in PAUP*. The initial tree topology does not seem to influence model selection, as long as the tree used is not a random topology (Posada & Crandall, 2001); neighbour joining was used only because it is a fast method to generate a 'better-than-random' tree. Likelihoods of the data for each partition were calculated using PAUP* under all standard nucleotide substitution models available in MrBayes v. 3.1.1. These likelihood scores were used to select a best-fitting substitution model using 'MrDT-ModSel', a modification of DT-ModSel (Minin *et al.*, 2003) developed by F.E.A. to compare only substitution models that are available in MrBayes v. 3.1.1 (Perl script available upon request to F.E.A.). For each dataset or partition, models were evaluated by using all sites or only variable sites as estimates of sample size (Posada & Buckley, 2004). When these different estimates of sample size caused MrDT-ModSel to select different models for a given data partition, we chose the model with fewer parameters. Four Bayesian analyses, each consisting of one cold and three heated Metropolis-coupled Markov chains, were run simultaneously in MrBayes v. 3.1.1, with random starting trees and trees sampled every 1,000 generations. A topological similarity criterion (the average standard deviation in partition frequency values across independent runs) was used to automatically assess convergence of the runs. When this value reached 0.01, the runs were terminated. Upon topological convergence, the first 25% of trees from each run were removed as burn-in. The postburn-in trees from all four runs were assumed to be independent samples from the posterior probability distribution, and thus were combined to produce a phylogram and a 50% majority-rule consensus tree.

For the multigene analyses, the data were partitioned in three ways: no partitioning (i.e. only one substitution model was used), partitioning by gene, or partitioning by gene and codon position. Partitioning by gene and codon resulted in four data partitions for the combined mtDNA dataset (a 16S rRNA partition and a partition for each COI codon position) and six for the three-gene dataset (16S rRNA, COI positions 1, 2 and 3, rhodopsin positions 1 + 2 and rhodopsin position 3; rhodopsin first and second codon positions were pooled due to low levels of variation). In analyses of the partitioned datasets, all model parameters except topology and branch lengths were unlinked across partitions. A rate multiplier was used for all partitioned analyses (the rate multiplier associates substitution rates for different partitions with a Dirichlet prior to allow different rates across partitions). Preliminary analyses suggested that the default temperature ($T = 0.2$) resulted in very few state swaps between chains, and some analyses were succumbing to the 'long tree' problem, in which estimated branch lengths were unreasonably long, as described by Marshall (2010) and Brown *et al.* (2010). To avoid these problems, analyses were performed with the temperature set to 0.05 (which resulted in state-swap frequencies of 60–70%) and the branch-length prior mean was reduced to 0.02 using the command 'brlens = unconstrained:Exp[50.0]', following the recommendations of McGuire *et al.* (2007) and Marshall (2010).

Appropriate partitioning schemes for the two multigene datasets were chosen using the AICc (a second-order correction of the Akaike Information Criterion) and the Bayesian Information Criterion (BIC), following McGuire *et al.* (2007) and using equations listed in Posada & Buckley (2004). These calculations required estimation of model likelihoods. The harmonic mean of likelihood values from the stationary phase of each analysis (calculated using the 'sump' command in MrBayes v. 3.1.2) was used as an estimate of the model likelihood, following Nylander *et al.* (2004). Estimated Bayesian posterior probabilities (BPPs) of clades on inferred trees were interpreted as measures of support.

RESULTS

Sequence data

We generated a total of 46 COI sequences and 43 rhodopsin sequences (Table 1). All individuals had unique COI

sequences, but several individuals had identical rhodopsin sequences; there were only 20 unique rhodopsin sequences. All of these sequences appear to be protein-coding sequences rather than pseudogenes; translation into amino acids using the flatworm mitochondrial genetic code (COI sequences) or the universal genetic code (rhodopsin sequences) in MacClade v. 4.08 (Maddison & Maddison, 2005) revealed no premature stop codons. A total of 141 out of 684 sites for COI were variable and 109 of these were parsimony-informative within *Sepia pharaonis*. By comparison, the rhodopsin data showed very low levels of variation. Only 10 of 523 sites for rhodopsin were variable within *S. pharaonis* (all but one of these sites were at the third codon position) and only seven of these sites were parsimony-informative.

Phylogenies

Phylogenies recovered in analyses of the COI and rhodopsin datasets were generally topologically concordant with one another and with phylogenies recovered from the combined analyses, so only the results of the analyses of the two dataset combinations (mtDNA and all three genes) will be discussed in detail and shown here. The rhodopsin phylogeny was poorly resolved due to the low level of variation found in this gene region among the focal taxa (tree not shown), but a monophyletic *S. pharaonis* comprising two subclades was recovered – one weakly supported subclade [BPP = 0.903, maximum parsimony bootstrap support (MPBS) = 53%] included all specimens collected in the Red Sea and Gulf of Aden plus one specimen ('N Gulf of Oman 5') collected from the Iranian coast of the Gulf of Oman, and a strongly supported subclade (BPP = 0.990, MPBS = 93%) comprising all other *S. pharaonis* and *S. ramani* individuals.

For both the combined mtDNA dataset and the three-gene dataset, the AICc and BIC values were lowest for the 'by gene and codon position' partitioning scheme, indicating that this was the best-fitting partitioning scheme of those evaluated for these data (Table 3).

The combined mtDNA phylogeny is shown in Figure 2. The phylogeny reveals five strongly supported subclades within *S. pharaonis*: a western Indian Ocean clade (Red Sea, Gulf of Aden and the northeast coast of Oman), a northeastern Australia clade (with a representative of '*S. ramani*' weakly supported as sister to this clade), an Iranian clade (northeastern Persian Gulf and northern Gulf of Oman), a western Pacific clade and a broadly distributed central Indian Ocean clade (west and east coasts of India and the Andaman Sea coast of Thailand). Relationships among these clades are somewhat poorly resolved, although there is some support for a clade comprising the Iranian clade, the western Pacific clade and the central Indian Ocean clade (BPP = 0.74, MPBS = 94%). One of the *S. ramani* specimens is nested within the central Indian Ocean clade, but the other is sister to the Australia clade. The Bayesian consensus phylogram does not clearly support monophyly of the *S. pharaonis* complex; sequences from *Metasepia tullbergi* and *S. lycidas* are part of a polytomy that includes all *S. pharaonis* specimens sampled in this study, although one resolution of this polytomy would have the *S. lycidas*/*M. tullbergi* clade as sister to a monophyletic *S. pharaonis* complex (an *S. pharaonis* sequence from GenBank is distantly related to the *S. pharaonis* sampled in our study, suggesting that the GenBank specimen was misidentified; this sequence was excluded from the three-gene dataset prior to analysis).

The three-gene phylogeny is shown in Figure 3. In contrast to analyses of the combined mtDNA data alone, the three-gene dataset gives some support for monophyly of the *S. pharaonis* complex (BPP = 0.70, MPBS = 81%) and a close relationship among the Iranian clade, the western Pacific clade and the central Indian Ocean clade (BPP = 0.96, MPBS = 93%). Both the combined mtDNA phylogeny and the three-gene phylogeny place a specimen denoted on the phylogenies as 'N Gulf of Oman 3*' (collected from the Iranian coast of the Gulf of Oman) within the western Indian Ocean clade with strong support. In contrast, 'N Gulf of Oman 5' (the specimen placed in the western Indian Ocean clade in the rhodopsin-only phylogeny) was recovered as a member of the Iranian clade in the three-gene phylogeny.

DISCUSSION

General patterns

As shown by Anderson *et al.* (2007), five strongly supported geographically delimited clades are evident on both the mtDNA and three-gene phylogenies. This study expands on the results of Anderson *et al.* (2007), however, in recovering moderate support for monophyly of the *Sepia pharaonis* complex, including a previously unsampled species (*S. ramani*), and in clarifying relationships among these five clades. The relationships among the *S. pharaonis* subclades are still not fully resolved, but some inferences can be made. The western Indian Ocean subclade appears to be sister to all of the other subclades (Fig. 3), and members of the western Indian Ocean subclade bear distinctly different rhodopsin sequences than nearly all other *S. pharaonis* sampled in our study. Although our data do not allow us to determine the precise location of

Table 3. The number of parameters, run length ('length') in millions of generations, best-fitting models, and AICc and BIC values for different partitioning schemes for the combined mtDNA (16S rRNA + COI) and three-gene (16S rRNA + COI + rhodopsin) datasets.

	mtDNA only (COI and 16S)			Three-gene (COI, 16S and rhodopsin)			
Partitions	None	Gene (C 16S)	Gene and codon (C1 C2 C3 16S)	None	By gene (C 16S R)	Gene and codon (C1 C2 C3 16S R12 R3)	Gene and codon (68 taxa)
No. of parameters	12	18	31	12	21	40	40
AICc	15197.83	15117.19	**14492.76**	12410.33	12186.68	**11592.84**	–
BIC	15258.09	15207.02	**14645.21**	12475.00	12298.91	**11802.58**	–
Models	GGI	GGI HG	GI F HG HG	GGI	GGI HG KG	GI F HG HG K HI	GI F HG HG K HI
Length	1.854	2.314	18.626	1.737	2.422	2.572*	8.382

The best (lowest) AICc and BIC scores are in bold text. AICc values shown were calculated using the number of variable characters; AICc values calculated using all characters were similar. Substitution model abbreviations are as follows: GGI = GTR = G = I, HG = HKY85 = G, HI = HKY85 = I, K = K2P, KG = K2P = G; see Anderson & Swofford (2004) for more information on model abbreviations and original citations for each model. Partition abbreviations are as follows: C, COI; R, rhodopsin; C1, COI position 1; C2, COI position 2; C3, COI position 3; R12, rhodopsin position 1 + position 2; R3, rhodopsin position 3. *Rerun for 50 million generations. The 68 taxa three-gene analysis was only run under the gene and codon partitioning scheme, and included the full set of taxa used in the combined mtDNA analyses.

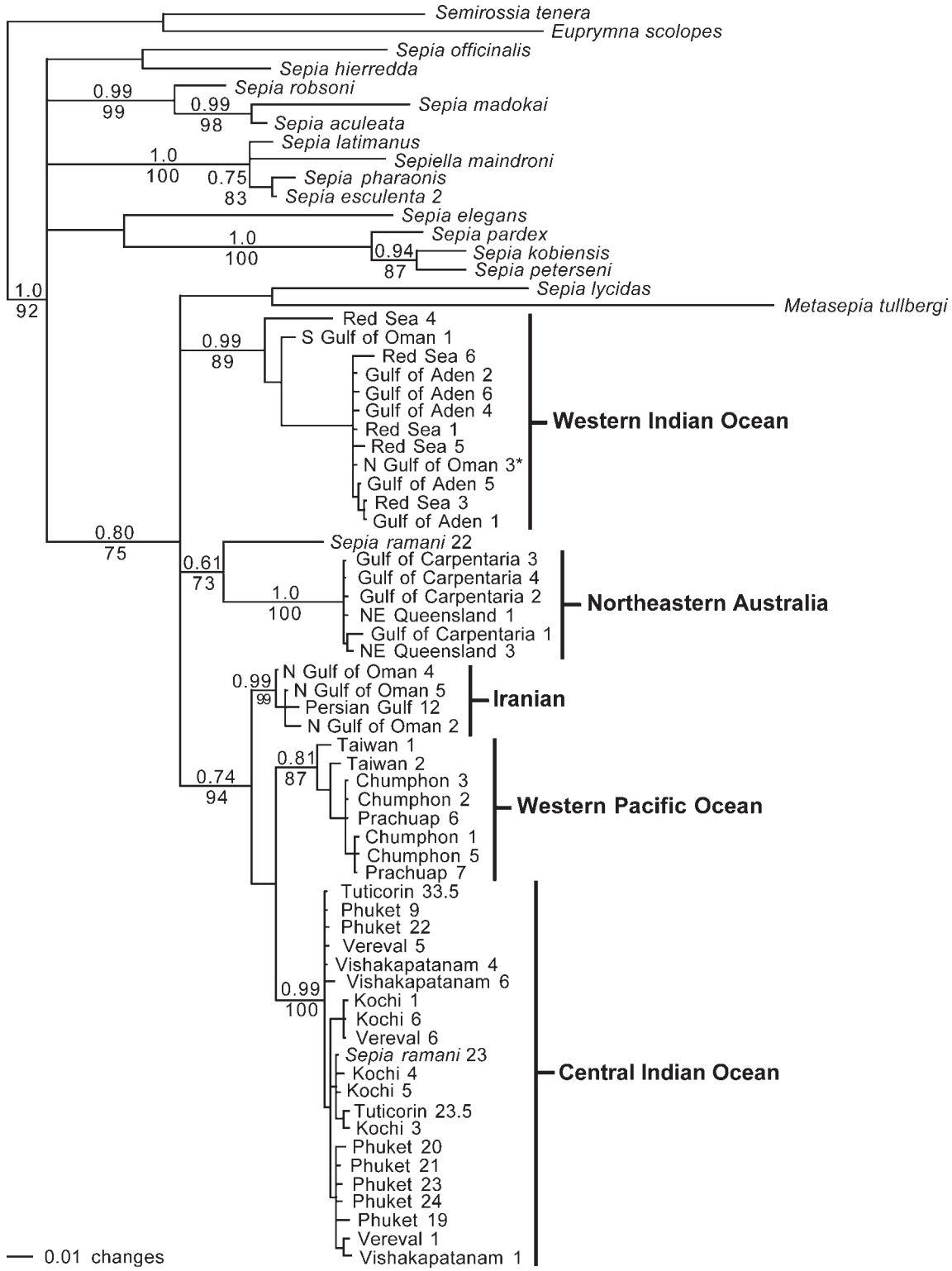

Figure 2. Fifty per cent majority-rule consensus Bayesian phylogram (branch lengths equal to the estimated number of substitutions per site averaged across all postburn-in trees) for the combined COI + 16S rRNA dataset, depicting the position of *Sepia pharaonis* haplotypes within Sepiidae and rooted with sequences from two sepiolid taxa. Numbers above branches are clade posterior probability (BPP) estimates; numbers below branches are MPBS values. Only nodes with BPP > 0.90 and/or MPBS > 70% have values associated with them, and support values within geographically delimited clades are not shown.

boundaries between phylogeographic units, it is clear that the regions where different clades are found differ substantially in size. One group of closely related individuals (the central Indian Ocean subclade) is distributed across the central Indian Ocean along the east and west coasts of India and the Andaman Sea coast of Thailand; in contrast, another group seems to be restricted to the Persian Gulf and northern Gulf of Oman (the Iranian subclade). Finally, it must be noted that

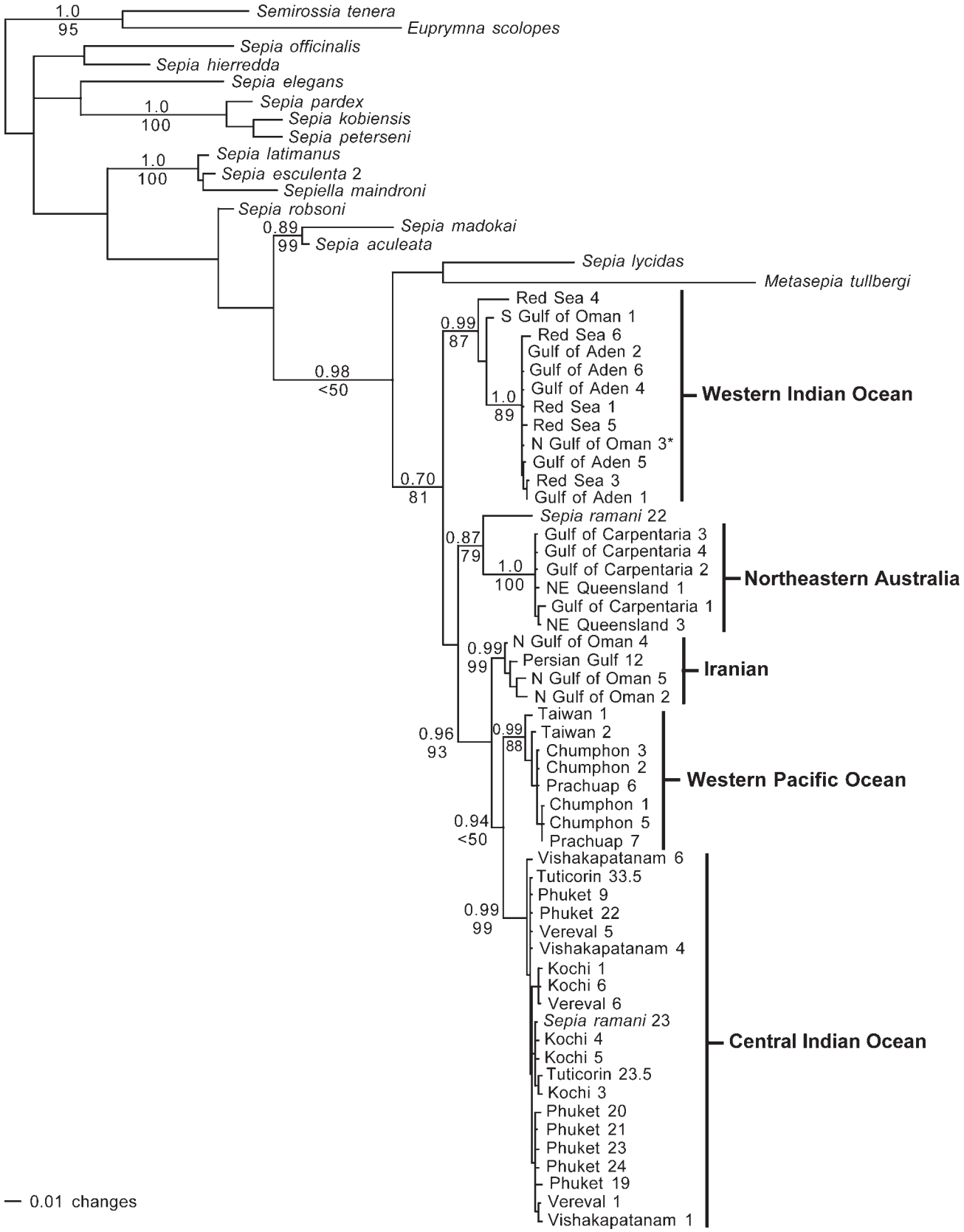

Figure 3. Fifty per cent majority-rule consensus Bayesian phylogram for the combined three-gene (COI + 16S rRNA + rhodopsin) dataset for the *Sepia pharaonis* complex. Support values associated with branches are as described for Figure 2.

representatives of only 14 sepiid species were used as outgroups in this study. Given that there are over 100 described species within Sepiidae (Khromov *et al*., 1998; Lu, 1998), our inference of monophyly for the *S. pharaonis* complex must be considered provisional pending sampling of additional sepiid species.

Concordance with other data

Sepia pharaonis shows considerable morphological and behavioural variation across its range, leading Norman (2000) to suggest that *S. pharaonis s. l.* consists of three forms: *S. pharaonis s. s.* (Red Sea to the Gulf of Oman, including the Persian Gulf), *Sepia pharaonis* II (western Pacific and northern Australia) and *S. pharaonis* III (Maldives to the Andaman Sea coast of Thailand). The western Indian Ocean clade revealed by Anderson *et al.* (2007) and confirmed here corresponds quite well to Norman's *S. pharaonis s. s.*, although we found evidence of a genetic break between the southern and northern Gulf of Oman (see below). Norman (2000) did not distinguish Iranian *S. pharaonis* from his *S. pharaonis s. s.*, and a photo in Norman (2000: 71) of a mating pair of *S. pharaonis* from Dubai (in the southern Persian Gulf) is used to demonstrate the zebra lines on the third arms that are supposedly diagnostic for *S. pharaonis s. s.* If *S. pharaonis s. s.* is equivalent to our western Indian Ocean subclade, this photo suggests that the Persian Gulf may be home to members of both our Iranian subclade and our western Indian Ocean subclade. Unfortunately, we lack the samples from the southern or eastern Persian Gulf that would allow us to test this possibility.

Our central Indian Ocean subclade may be Norman's *S. pharaonis* III, although we found that *S. pharaonis* individuals collected along the west coast of India from as far north and west as Veraval are also members of this clade (i.e. genetically, the range of this form extends north and west of the Maldives along the Indian coast). Finally, Norman's *S. pharaonis* II appears to comprise at least two genetically distinct groups: our western Pacific subclade (comprising samples from Taiwan and the Gulf of Thailand) and our northeastern Australia subclade. This finding was foreshadowed by Reid *et al.* (2005), who noted that hectocotylus morphology differs between *S. pharaonis* specimens from Japan (presumably members of our western Pacific subclade, though it is possible that Japan is home to yet another *S. pharaonis* subclade) and Australia. In short, two of our subclades correspond well to the forms described by Norman (2000), but we found that his *S. pharaonis* II represents at least two genetically distinct groups, and we have also found evidence for a distinct Iranian subclade.

The status of Sepia ramani

Sepia ramani is a neritic demersal southeastern Indian species that is morphologically very similar to *S. pharaonis*, and there has been some controversy regarding the status of *S. ramani* as a distinct species. One specimen that we sequenced (*S. ramani* 23) is a member of the central Indian Ocean subclade of *S. pharaonis* (Figs 2, 3). This specimen could be misidentified, or it could be a hybrid (or backcross) between *S. ramani* and *S. pharaonis* that exhibits *S. ramani* morphology but carries a *S. pharaonis* mtDNA haplotype. By contrast, the other *S. ramani* specimen collected from the same area (*S. ramani* 22) is genetically distinct from all other specimens collected from Indian waters. Surprisingly, it does not group with the central Indian *S. pharaonis* subclade; it groups with the northeastern Australia subclade, although it is quite distinct even from the latter subclade. Our analyses suggest that *S. ramani* is part of the *S. pharaonis* species complex, but that *S. ramani* 22 may represent a distinct subclade within the complex.

Gene flow between regions

There is generally little evidence of migration of cuttlefish between geographic regions in our data. However, two of the four specimens collected in the Gulf of Oman (N Gulf of Oman 3* and N Gulf of Oman 5) showed discordance between clade membership and collection locality. N Gulf of Oman 3* grouped strongly with the western Indian Ocean subclade on all phylogenies, while N Gulf of Oman 5 grouped with the Iranian subclade (as expected) in the mtDNA and three-gene phylogenies (Figs 2, 3), but with the western Indian Ocean subclade on the rhodopsin phylogeny. The latter finding suggests that the mtDNA and rhodopsin sequences for N Gulf of Oman 5 are in conflict, and that phylogenetic signal from the mtDNA overwhelmed the signal from the rhodopsin data for this specimen in the combined analyses. The geographic regions in question are adjacent to one another; one member of the western Indian Ocean subclade (S Gulf of Oman 1) was collected from the southern coast of the Gulf of Oman, while the Iranian specimens (N Gulf of Oman 2, 3, 4 and 5) were collected about 230 km to the northeast, on the opposite side of the Gulf of Oman.

In this case, our samples seem to have come from at or near a boundary between two subclades, and we are detecting either migrants or individuals resulting from crosses or backcrosses between members of these two subclades (e.g. N Gulf of Oman 5, whose mtDNA haplotype is Iranian but whose rhodopsin sequence appears to be from the western Indian Ocean). Our only specimens from the Persian Gulf are members of the Iranian subclade, but we do not know if members of the western Indian Ocean subclade are also found in the Persian Gulf, so we cannot discern whether the boundary between these two subclades is in the Gulf of Oman or the Persian Gulf (or both). The Gulf of Oman ranges from 60 km (at the Strait of Hormuz) to 370 km wide (from Ras Al Hadd, Oman to Gwadar Bay, Pakistan) and the Gulf of Oman basin is about 3,400 m deep (Uchupi, Swift & Ross, 2002). *Sepia pharaonis* is a neritic demersal species so direct dispersal across the Gulf of Oman seems unlikely. Allcock *et al.* (1997) found that a distance of only 30 km of deep ocean severely limits larval dispersal in *Pareledone turqueti* (Joubin 1905), an Antarctic octopus. However, currents might facilitate rare dispersal events across the Gulf of Oman at certain times of the year. During the southwestern (summer) monsoon, the Ras Al Hadd jet (a continuation of the Somali and Oman Coastal Currents that flows eastward from the eastern tip of Oman; Schott & McCreary, 2001) and the cyclonic eddy it produces in the Gulf of Oman could promote occasional dispersal of *S. pharaonis* juveniles across the Gulf. However, the main spawning season for *S. pharaonis* in this region is November and December, between the monsoons (Reid *et al*., 2005) and after the Ras Al Hadd jet has weakened.

Biogeography

This investigation of *S. pharaonis* phylogeography may shed some light on biogeographic patterns of neritic animals in the Indian Ocean and western Pacific. Comparisons with other studies are somewhat compromised by the fact that although numerous phylogeographic studies of Indian Ocean species have been published, the Malay Archipelago, South Africa and Australia have received substantially more attention than the northern Indian Ocean (i.e. the coasts of south Asia, the Arabian Peninsula and northeastern Africa). Furthermore, within the Indian Ocean, archipelagos with extensive reef systems such as Seychelles, Mauritius and the Maldives also seem to have been sampled more frequently than the continental shelves of south Asia and northeastern Africa. The focus on

species or species groups that span the boundary between the Indian Ocean and Pacific Ocean (the 'marine Wallace's Line'; Barber *et al*., 2000) is understandable, given the importance of this region in both marine and continental biogeography, but it does not provide much insight into Indian Ocean phylogeography. Despite this bias, there are several phylogeographic studies whose focal taxa are found in many of the same regions where *S. pharaonis* is found, and comparisons with these studies may be fruitful.

Recovery of a sister pair consisting of the Western Pacific clade and the Central Indian Ocean clade in the *S. pharaonis* complex, though weakly supported (BPP = 0.94, MPBS < 50%), is consistent with numerous other studies that have found similar sister species or population pairs, with one species (or population) in the Indian Ocean and the other in the Pacific Ocean (Williams *et al*., 2002 and citations therein; Reid *et al*., 2006). Genetic divergence between Indian and Pacific populations of marine species has been attributed to reductions in gene flow during repeated periods of glaciation over the last 140,000 years, which resulted in lower sea levels, reduced transfer of warm surface water between the Indian and Pacific Ocean basins and increased cold-water upwelling as recently as 18,000 years ago (Potts, 1983, 1984; Fleminger, 1986; Springer & Williams, 1990; Williams *et al*., 2002). It is possible that Pleistocene glaciations also played a role in the divergence between the central Indian Ocean and western Pacific clades of *S. pharaonis*, though the current lack of divergence time information for the *S. pharaonis* complex limits our ability to test hypotheses of causality.

Though less work has been done on western Indian Ocean marine populations, some studies have found evidence of a phylogeographic break between the eastern and western Indian Ocean (Ridgway & Sampayo, 2005). The deepest divergence within the *S. pharaonis* complex is between the western Indian Ocean clade and the rest of the complex. Within the western half of the Indian Ocean, three *S. pharaonis* clades were found, with a possible boundary between the Iranian clade and the western Indian Ocean clade in the Gulf of Oman. A similar pattern has been found in *Lunella coronata*, a gastropod found on rocky shores from southeastern Africa through the Gulf of Oman to the western Pacific (Williams *et al*., in review).

Differences in phylogeographic patterns across studies of Indo-Pacific neritic taxa are not surprising, given the substantial differences in life history, ecology and behaviour among these taxa. Crandall *et al*. (2008) found that phylogeographic patterns can differ substantially between sympatric species, even when those species are congeneric and ecologically similar. Furthermore, phylogeographic studies of Indian Ocean marine fauna encompass taxa of differing ages, which may have been impacted by different vicariant events or paleooceanographic phenomena (Page, 1990, 1991). Conversely, phylogenetic patterns may be concordant across taxa, but these similarities could be due to pseudocongruence, in which similar phylogenetic patterns arise among two or more taxa of different ages that were affected by different vicariant events (Cunningham & Collins, 1994; Donoghue & Moore, 2003).

At present, we have no divergence time information for clades within the *S. pharaonis* complex. All hope is not lost, however, because sepiids possess a calcified structure that would seemingly be amenable to fossilization – the cuttlebone. Furthermore, the cuttlebone of *S. pharaonis* has a distinctive cuplike extension covering the striated zone of the posterior inner cone (Khromov *et al*., 1998; Norman, 2000), which may allow fossil members of the *S. pharaonis* complex to be identified. Unfortunately, fossil cuttlebones are quite rare; the structure and composition of cuttlebones make them less likely to occur as aragonitic fossils than nautiloid or ammonite shells, and postmortem destruction of drifting cuttlebones may severely limit deposition in the first place (Hewitt & Pedley, 1978). To our knowledge, no fossil cuttlebones attributable to *S. pharaonis* have been found. Recovery of cuttlebones attributable to the *S. pharaonis* complex might allow estimation of the age of the complex and divergence times within the complex, allowing phylogeographic comparisons of *S. pharaonis* with other neritic species in the Indian Ocean.

Taxonomy

Our results show that *Sepia* '*pharaonis*' is a complex of at least five subclades (and perhaps six, depending on the status of *S. ramani*). As Anderson *et al*. (2007) noted, the type localities of *S. pharaonis* are both in the Red Sea (near El-Tor in the Sinai in the northern Red Sea and near Massawa in Eritrea along the west coast of the Red Sea). Though we did not obtain samples from the type localities, we did obtain samples from the Yemeni Red Sea coast (340 km east of Massawa) and found that these specimens were members of our western Indian Ocean subclade. In light of this, we believe that specimens from the type localities would probably be members of our western Indian Ocean subclade. This subclade is sister to a clade comprising all other subclades in the complex (including *S. ramani*; Fig. 3), and the distinction between this subclade and all other subclades in the complex is the only distinction that is supported by the rhodopsin sequence data. In light of this, we suggest that the binomen *S. pharaonis* be restricted to the western Indian Ocean subclade.

Sepia ramani is a member of the *S. pharaonis* species complex, though one of our *S. ramani* samples may represent an additional, previously unsampled subclade within the complex. The taxonomic status of *S. ramani* (as well as usage of the binomen *S. pharaonis* itself) hinges on the taxonomic status of the five unnamed subclades, and this cannot be fully addressed without detailed morphological and morphometric work, preferably coupled with additional genetic data collection to provide a link with our study. Furthermore, additional specimens from as-yet-unsampled parts of the range of the *S. pharaonis* complex must be evaluated, as there may be additional subclades (or species) waiting to be discovered; regions of particular interest are Madagascar, the Philippines, the Yellow Sea (Hwang Hai) and Indonesia.

ACKNOWLEDGEMENTS

This research was supported by NSF Grant DEB-0235794 to F.E.A. We thank Alonso Córdoba, Stephanie Clutts, Mike Venarsky and Adria Pilsits for assistance with DNA extraction, PCR and sequencing and Associate Editor Suzanne Williams and two anonymous reviewers for their very helpful comments and suggestions.

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